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FINAL REPORT

Natural Selection of Microorganisms in Extreme Environments

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The present program is concerned with the basis for the natural selection of microorganisms exposed to two environmental extremes: drought and starvation. To some extent, the extreme of salinity has also been considered. The program was supported by residual funds remaining at Cornell University following the departure of Dr. J. R. Vallentyne, Principal Investigator on a program entitled "Paleobiochemistry of amino acids and polypeptides."

Most terrestrial microorganisms which are capable of surviving for long periods in a desiccated or starved condition form resistant bodies such as endospores, cysts, chlamydospores or sclerotia. However, there are certain bacteria which, though unable to form endospores, can also survive for long periods of drought or nutrient deficiency. Although occasional reports have been concerned with the decline in viable bacteria during the drying of terrestrial soils, explanations for the persistence of these long-lived species and qualitative and quantitative studies of microbial survival in such environments are few. In the present study, bacteria susceptible and resistant to extreme drought were isolated and characterized, and the ability of each to survive under environmental stress was examined.

Two soil samples, a Collamer silt loam and a Honeoye silt loam, were collected and immediately passed through a 1 mm diameter sieve. Five grams of each soil were placed in 35 x 10 mm diameter petri dishes. Replicate dishes for each sample were incubated in a desiccator over calcium chloride, and soil samples were removed at regular intervals for the enumeration of viable bacteria. Microbial counts were performed using conventional techniques on soil extract agar, asparagine-mannitol agar and nutrient agar containing 1:500,000 crystal violet. Representative colonies having different

morphological characteristics were selected and examined microscopically.

Bacteria isolated from these soils were grown at 30° on a rotary shaker in flasks containing either nutrient broth or, for Rhizobium, a yeast extract-mannitol solution. The cells were collected during the stationary phase of growth by centrifugation and washed three times in phosphate buffer. Six grams of sterilized quartz sand were placed in petri dishes and moistened uniformly with the bacterial suspension. The total number of viable cells applied to each dish was approximately 2×10^8. Replicate dishes of each organism were placed in a desiccator over calcium chloride and samples taken at regular time intervals. The survival of these organisms was determined by plate dilution techniques using either nutrient agar or yeast extract-mannitol agar.

A number of bacteria were isolated during the period of drought stress. These organisms were tested for heat resistance (survival at 85° for ten minutes) in order to exclude the spore formers, and they were repeatedly examined under the microscope in order to eliminate from consideration the actinomycetes, whose persistence is attributable to the formation of drought-tolerant conidia. Those nonspore-forming and nonconidia-forming organisms capable of surviving the drought stress for more than fifteen days were considered as resistant and those that perished within one to four days were designated as susceptible. In addition, three Rhizobium strains were employed.

In studies of the effect of osmotic pressure on the ability of bacteria to withstand dryness, five resistant and five susceptible organisms were selected. The susceptible forms included a Flavobacterium, a Pseudomonas, a

Rhizobium and two gram negative rods. The resistant forms included one Arthrobacter strain, 1 gram positive short rod and 3 different kinds of gram negative short rods. These bacteria were cultured in growth media containing a mixture of NaCl, KCl and Na_2SO_4 in a molar ratio of 5:3:2. Quantities of these salts were added to the medium to obtain the desired a_w (water activity). The basal medium was adjusted to an a_w level of 0.999. Comparisons of the survival of bacteria to extreme drought were made after the cells were grown in the basal medium and in a medium with an a_w just allowing for growth of the culture. The cells were washed three times with a solution having the same a_w as the growth medium and suspended in a salt solution with a_w value of 0.995. The initial numbers of cells exposed to the various osmotic tensions were adjusted turbimetrically so that the population densities were essentially identical. The survival of these bacteria was determined by the usual methods with the counts being taken at periods ranging from 0 to 25 days.

To determine the minimum a_w value for growth, the various microorganisms were cultured in tubes containing nutrient broth or yeast extract-mannitol medium with varying a_w values. The cultures were incubated for seven days, and the appearance of growth in the various solutions recorded.

Extensive studies were conducted on the die-away and on the changes in morphological types of terrestrial bacteria. The vast majority of microorganisms, regardless of morphological characteristics, were found to be quite drought susceptible. However, certain gram negative rods and gram positive cocci were capable of maintaining viability for periods ranging up to 60 days, or occasionally longer. Representatives of the genus Arthrobacter were par-

ticularly resistant to dryness, and they survived for periods of three months or longer. The actinomycetes and members of the genus Bacillus were universally abundant in the resistant community. Nevertheless, the total number of individuals of these two groups declined rapidly during the initial period of stress, but this period of rapid decline was followed by a constant population density; the reason for the rapid die-away and subsequent constant population level is probably associated with the inactivation of vegetative cells and the persistence of the Bacillus endospores or Streptomyces conidia. The soil extract medium gave higher total counts and a larger variety of microorganisms. Nutrient agar containing crystal violet gave a low total count but supported the development of a high percentage of gram negative bacteria. Asparagine-mannitol agar supported a high percentage of actinomycetes and nonfastidious bacteria.

The resistance to drought stress was affected by the age of organism introduced into the stress circumstances. For example, the population of one organism, when obtained from a 17-hour culture, contained 2500 survivors after a 20-day drought period, while the same organism obtained from a 144-hour culture yielded less than 10 survivors. The initial population density in both instances was approximately 10^8.

Striking results were obtained when the lower limits of a_w that permitted growth of the drought-resistant and drought-susceptible organisms were determined. Of the 17 resistant bacteria, 10 were capable of growing in a medium with an a_w value of 0.960 or lower, whereas only 2 of the 15 susceptible bacteria were capable of multiplying in such solutions. For 5 of the 15 susceptible bacteria, the minimum a_w was 0.995 although all 17 of the resistant forms

were capable of multiplying in media of lower a_w activities. Thus, there seems to be a marked selection, associated with resistance to drought stress, for organisms able to grow in solutions of high osmotic pressure.

Moreover, when 5 susceptible bacteria were grown in media of higher than usual osmotic pressure, presumably leading to an increase in the internal osmotic tension of the cell, these cells acquired the ability to survive longer in extremely dry environments. In some instances, remarkable increases in the resistance were found when these sensitive organisms were grown in media of low a_w as contrasted with the same population cultured in solutions of high a_w. When the resistant bacteria were grown in media of low a_w, either there was no effect in increasing their drought resistance or they too became still more resistant to dry conditions.

The ability to withstand extreme dryness was also increased after bacteria were adapted, either by a mutation or by a nongenetic change in the population, to grow in media with low a_w values. Thus, drought-susceptible Flavobacterium sp. and a drought-susceptible gram-negative rod, adapted to solutions of high a_w were capable of surviving far longer than the initial populations from which these variants were derived. The lower the a_w value of the solution in which these microorganisms were grown, the more capable was the population of withstanding drought stress.

Studies have also been conducted on the survival of terrestrial microorganisms and the changes of populations in a microbial community during starvation stress. The soil samples, the same as previously employed, were added to a solution containing inorganic ingredients (KNO_3, K_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$) but no carbon. The microbiological changes occurring in this

carbon-limited solution were determined at regular intervals during a 330-day period. The viable population that disappeared and new organisms that appeared during the course of time were enumerated on the three media previously used. The morphological and colonial characteristics of the various organisms that developed on these plates were recorded.

Pure cultures of bacteria were isolated and the heat-resistant microorganisms and the actinomycetes were eliminated from concern because the spores of Bacillus and the conidia of the actinomycetes are known to be starvation-resistant. In addition, two strains of Rhizobium trifolii, which were variants of the same parent culture and serologically identical, were employed. The bacteria were grown in either nutrient broth or in yeast extract-mannitol media, and the bacteria were collected when in the stationary phase of growth. The cells were then washed aseptically, and between 10^6 and 10^7 cells were inoculated into a flask containing the carbon-free solution. Viable cell counts were then determined. Two groups of organisms were readily differentiated by these means: susceptible cells which were killed within 10 days and resistant forms that persisted in excess of 24 days during the starvation stress. Four of the susceptible and four of the resistant bacteria were obtained in pure culture. The four resistant bacteria were a Micrococcus, a gram-negative rod and two different short gram positive rods. The resistant forms included three different gram negative rods and a gram positive rod.

Extensive data were obtained on the microbiological changes occurring in the terrestrial community exposed to starvation stress in this carbon-free solution. Initially, a large number of various pigmented or nonpig-

mented gram negative rods and a group of gram positive cocci were observed. As the period of starvation progressed, the total population density of bacteria declined. However, a period of cryptic growth was noted after about 15 to 30 days and 150 to 200 days in one of the two soil-derived communities. As expected, a high percentage of the resistant cells were either members of the genus Bacillus or actinomycetes of the genus Streptomyces. The percentage of the spore-forming bacteria increased with prolonged starvation.

When isolates that were obtained after prolonged drought were compared with isolates derived from populations that disappeared readily during starvation stress, all resistant bacterial strains were found to be able to survive for more than 24 days of starvation while all of the susceptible bacteria perished within less than 10 days. Likewise, the two cultures of Rhizobium trifolii, though obtained from the same initial clonal population, differed markedly in the ability to survive this form of environmental stress.

A comparison was made of the endogenous respiration of both groups of bacteria. The endogenous respiratory rates of the resistant bacteria were either quite high or reasonably low. The endogenous respiratory rate of the starvation-susceptible bacteria ranged from low to high. No correlation was apparent between the ability of the organisms to persist during starvation and their endogenous activity. The utilization of PHB reserve polymer and glycogen by the culture was investigated too. The consumption of PHB was found to be more rapid during the initial periods of starvation and the level fell to a steady value subsequently.

All starvation-resistant bacteria were found to contain a high content of PHB (34-300 mg/gram dry weight) whereas the PHB level in the susceptible bacterial cells was found to be uniformly very low (0.06-3.4 mg/gram dry weight). An exception is R. trifolii, which did not behave like the other susceptible organisms in regard to PHB content. The glycogen concentration in the cells was found to be low in all of the starvation-resistant bacteria but also in most of the susceptible strains.

In investigations of the microorganisms of saline extremes, 27 halophilic bacteria were isolated from various environments and 4 were examined in detail. In order to obtain a method for ascertaining the number of each species at various stages of the succession taking place in a mixed saline community, studies were conducted to test the resistance or susceptibility of these bacteria to various physical and chemical treatments, and a method was developed by which it is possible to differentiate among the test isolates in mixed culture. These studies have not been completed, but funds have been obtained from other sources to continue this aspect of the study. In addition, an investigation is being continued, as an extension of this NASA-supported program, of the succession occurring in model terrestrial saline communities.